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Rapid report

Disc formation in cholesterol-free liposomes during phase transition ☆

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Abstract

Cryogenic transmission electron microscopy (cryo-TEM) images of lysolipid-containing thermosensitive liposomes (LTSL) revealed that open liposomes and bilayer discs appeared when liposomes were cycled through the gel ($L_{\beta'}$) to liquid-crystalline (L_{α}) phase transition. The amount of bilayer discs generated was dependent on the combined presence of PEG-lipid and lysolipid in the membrane. We hypothesize that micelle-forming membrane components stabilize the rim of bilayer openings and membrane discs that form when liposomes are cycled through T_C.

Keywords: Thermosensitive liposome; Lysolipid; PEG-lipid; Disc formation

Several laboratories have investigated the use of cholesterol-free or low cholesterol-containing liposomes exhibiting a melting phase transition temperature in the range of 41 °C–43 °C to achieve hyperthermia-triggered release of encapsulated drugs for enhanced therapeutic activity [1]. Early liposome formulations were composed of dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC) in the absence of cholesterol [2,3] or DPPC and DSPC combined with PEG-derivatized phosphatidyl ethanolamine lipids (PEG-lipids) [4] and small amounts of cholesterol [5,6]. These liposomes typically exhibited minimal drug release at or below physiological temperatures but released their encapsulated contents upon increasing the temperature to the gel ($L_{\beta'}$) liquid-crystalline (L_{α}) phase transition temperature (T_C) over a period of approximately 30 min [1]. The release mechanism in such systems is based on increased membrane permeability during coexistence of membrane areas in $L_{\beta'}$ and L_{α} phases [7].

The use of these traditional thermosensitive liposomes (TTSL) in combination with local hyperthermia for target-specific delivery of anticancer drugs such as doxorubicin to solid tumors has been shown to result in increased antitumor activity relative to conventional nonthermosensitive liposomes and free drug [1]. More recently, the release rate of the anticancer drug doxorubicin from liposomes at the T_C of the formulation could be increased dramatically by incorporation of 10 mol% lysolipid in the liposomal membrane [8]. Characterization of the temperature dependence of the drug release kinetics in lysolipid-containing thermosensitive liposomes (LTSL) revealed that maximum drug release rates were obtained approximately 1 °C below the T_C of DPPC, which led to a postulated release mechanism based on premelting of grain boundaries in gel phase phospholipid membranes. It was speculated that by heating to temperatures just below the T_C, lysolipids were enabled to leave the membrane from grain boundaries, creating structural defects, through which encapsulated drugs could rapidly permeate [9]. In this study, we examined morphological features of LTSL and TTSL using cryogenic transmission electron microscopy (cryo-TEM) before and after cycling liposomes through their

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T_C in order to gain insight into structural changes of the LTSL membrane during $L_{\beta'}$ to L_α phase transition.

Cryo-TEM images of liposomes were taken before and after repetitively cycling samples through T_C by heating and cooling liposomes to temperatures of 50 and 22 °C, respectively, for 10 min. Before T_C cycling, liposomes exhibited polyhedral shapes with faceted surfaces and this appearance was more pronounced in liposomes with smaller diameters (Fig. 1A). When LTSL were cycled one time through T_C after extrusion, some open liposomes and bilayer discs appeared (Fig. 1B). After five T_C cycles, the amount of open liposomes and bilayer discs increased considerably. Bilayer discs were smaller than the liposomes from which they were derived, with an average size of approximately 50 nm (Fig. 1C).

Based on our hypothesis that micelle-forming membrane components are responsible for disc formation during phase transition, the contribution of the two LTSL membrane components capable of forming micellar aggregates, namely DSPE-PEG_{2000} and the lysolipid MSPC, was investigated. In the LTSL sample without PEG-lipid, bilayer discs could not be detected before (Fig. 2A) or after (Fig. 2B) cycling liposomes five times through their T_C. In the LTSL sample without MSPC, bilayer discs were not present initially (Fig. 2C) but appeared after cycling five times through T_C (Fig. 2D). Although discs appeared in this sample after T_C cycling, the amount of bilayer discs after T_C cycling was less than in liposomes containing both PEG-lipid and MSPC together (compare to Fig. 1C).

The appearance of small cholesterol-free phospholipid liposomes below T_C is generally spherical with a faceted surface, as revealed herein by cryo-TEM imaging and previously by other research groups [10,11]. The origin of this polyhedral morphology likely stems from the presence of individual gel phase plates (domains) separated by grain boundaries. Grain boundary formation occurs in the $L_{\beta'}$ phase of phospholipid membranes when the temperature is decreased from values above to values below the T_C of the

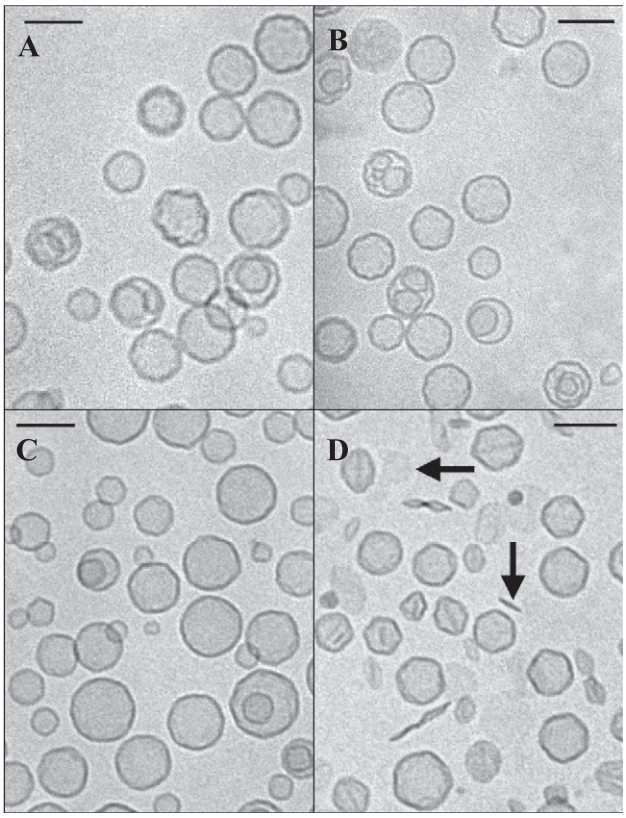

Fig. 2. Cryo-TEM images of freshly prepared LTSL without DSPE-PEG_{2000} (DPPC/MSPC, molar ratio, 90:10) before (A) and after (B) cycling five times through T_C and LTSL without MSPC (DPPC/DSPE-PEG_{2000}, molar ratio, 90:4) before (C) and after (D) cycling five times through T_C. Black arrows indicate bilayer discs. Scale bars indicate 100 nm.

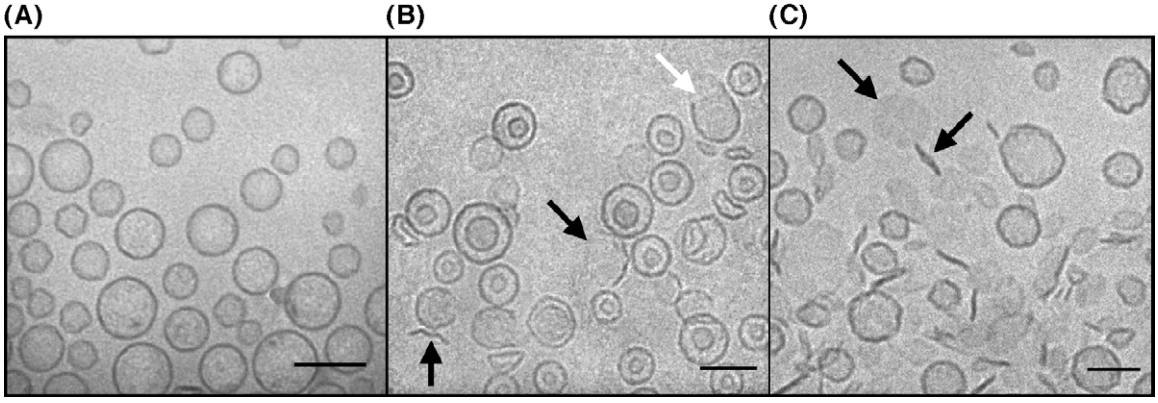

Fig. 1. Cryo-TEM images of LTSL (DPPC/MSPC/DSPE-PEG_{2000}, molar ratio, 90:10:4) before and after cycling through T_C after extrusion. Freshly prepared LTSL were vitrified from a temperature of 22 °C (A), 50 °C (B), or after cycling liposomes five times through their T_C (C). Liposomes were prepared by the method of lipid film hydration and extrusion as described by Hope et al. [23]. Cryo-TEM images were taken on a Zeiss EM 902 A transmission electron microscope in zero-loss bright-field mode and an acceleration voltage of 80 kV. Liposomes were diluted to a lipid concentration of 10 mM and transferred onto a copper grid coated with holy polymer film at controlled temperature and humidity conditions. Excess sample was removed by aspiration onto filter paper. The thin (10–500 nm) sample films were vitrified by rapid submersion in liquid ethane and transferred into the microscope at a temperature of −165 °C. Details of the procedure are described elsewhere [24]. White arrows indicate open liposomes, black arrows indicate bilayer discs. Scale bars indicate 100 nm.

membrane [12,13]. Upon cooling, initially multiple $L_{\beta'}$ phase nuclei form in the unordered L_{α} phase membrane. These nuclei with a high degree of order grow in size as the temperature decreases until eventually the entire membrane is in the $L_{\beta'}$ phase. The degree of disorder within the $L_{\beta'}$ phase membrane is highest at domain-separating boundaries (grain boundaries) because the lattice orientation of $L_{\beta'}$ phase domains is different in each domain [12]. As modeled by Mouritsen and Zuckermann [14,15], the T_C of the phospholipid membrane at grain boundary areas is slightly lower than that of membrane domains and thus, phospholipids at grain boundaries melt at temperatures a few degrees below the T_C of the bulk lipid.

Grain boundaries with a high degree of defects are predispositioned to accommodate a high membrane curvature. Consequently, liposomes with grain boundaries are likely to adapt a polyhedral shape with flat, highly ordered gel phase domains intersected by boundary regions with a higher degree of disorder and curvature. The degree of curvature at boundary regions increases with decreasing liposome size in response to increasing bending forces and can be annealed by increasing the temperature above the T_C of the liposome membrane [16].

Our and previous [11] results indicate that the morphology of liposomes below their T_C is dependent on the liposome size. Thus, cholesterol-free liposomes are less likely to adapt a faceted polyhedral morphology when greater in size than approximately 100 nm. This difference in liposome morphology may reflect the difference in strength of membrane bending forces or the fact that the faceted structure may not be apparent in cryo-TEM images because the angles between bilayer plates increase with increasing liposome size.

Lysolipids have been shown to segregate from the bulk lipid by lateral phase separation [17–19]. This segregation of membrane components has been distinguished from classical lateral lipid phase separation and named lateral lipid domain formation and was shown to be dependent on the degree of bilayer curvature and thus on the liposome size [20]. We propose that in small (100 nm) LTSL, micelle-forming membrane components such as PEG-lipids and lysolipids accumulate in 100-nm liposomes at grain boundaries and occupy structural defects in the membrane.

Based on the shape concept [21,22] the thermodynamically most favoured aggregate of phospholipid molecules depends on the ratio between their acyl chain tail volume (v) and the product of headgroup area (a_0) and tail length (l_c), which calculates a shape factor $N_s=v/a_0\times l_c$. For PEG-lipids and lysolipids with a shape factor $N_s<1/3$, the thermodynamically most favoured aggregate in aqueous solution is the spherical micelle or the cylindrical micelle, whereas phosphatidylcholines such as DPPC with $N_s\approx 1$ aggregate into bilayers. Assuming that micelle-forming membrane components accumulate at grain boundaries and that grain boundaries melt prior to the bulk lipid, micelle-forming membrane components such as DSPE-PEG_{2000} and MSPC could adopt a thermodynamically favoured micelle-like conformation within the bilayer upon melting and thereby stabilize the rim of open liposome structures. Depending on the degree of lateral lipid domain formation, this process may lead to the formation of membrane pores, open liposomes, or bilayer discs (Fig. 3).

The fact that membrane discs were not observed in LTSL without DSPE-PEG_{2000} suggests that DSPE-PEG_{2000} is needed to sterically stabilize open liposome structures and prevent their closure or fusion with other membranes. Our

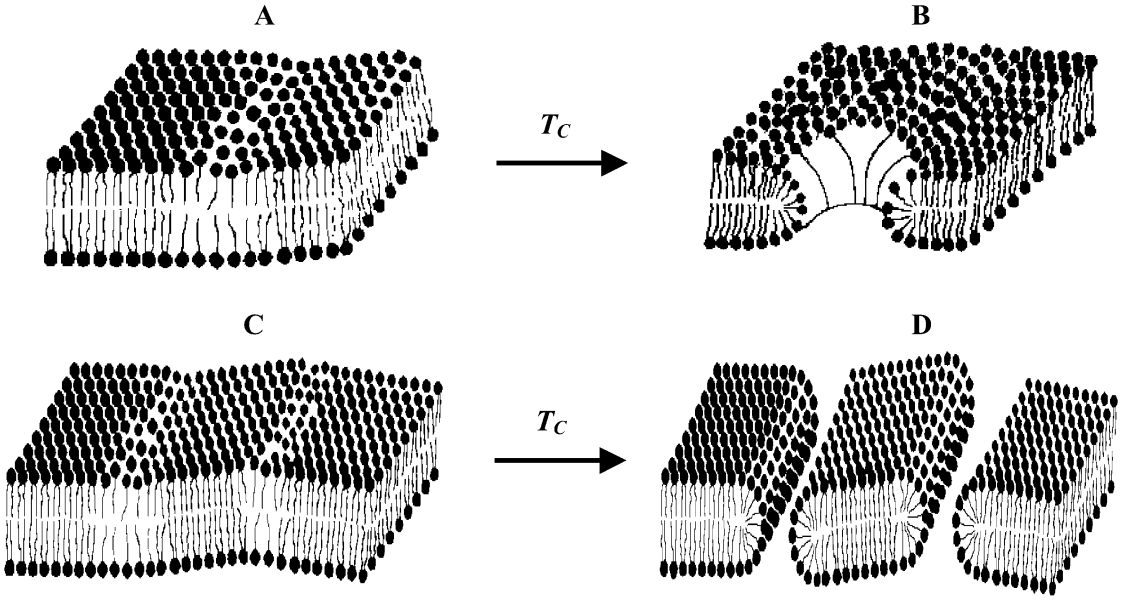

Fig. 3. Schematic representation of proposed pore- and disc-formation in LTSL. (A, C) Accumulation of micelle-forming lipids at grain boundaries. (B) Pore formation during phase transition. (D) Disc formation during phase transition. PEG-lipids are not included in the drawing, T_C=phase transition temperature.

results indicate that the presence of both membrane components, DSPE-PEG_{2000} and MSPC, is necessary to generate a significant proportion of membrane discs after T_C cycling. It appears that in LTSL, DSPE-PEG_{2000} functions in a similar capacity as MSPC and its ability to accommodate a high surface curvature at the rim of open bilayer structures is augmented by the presence of lysolipid in these membrane areas. Thus, the presence of PEG-lipids and lysolipids in the liposome membrane, especially if segregated into grain boundaries, could facilitate the temperature-triggered formation of membrane pores, liposome openings, and the disintegration of liposomes into membrane discs. It should be noted that significant disc formation only occurred after multiple cycles through T_C, whereas drug release from LTSL occurs within seconds of heating liposomes to 1 °C below their T_C. Consequently, although disc formation occurs in these liposomes, it is unlikely to be directly responsible for the release of drug upon heating. However, structures that must form as precursors to disc formation may be sufficient to allow rapid efflux of entrapped contents during phase transition via pore formation.

In summary, cycling cholesterol-free thermosensitive liposomes throughout their T_C in the presence of DSPE-PEG_{2000} and MSPC in the membrane results in the formation of open liposome structures and membrane discs. We have presented evidence that pre-formation of microstructures in the $L_{\beta'}$ phase of the liposome membrane results in a temperature-triggered thermodynamically favoured reorientation of phospholipid molecules in the LTSL membrane at their T_C, which can lead to a temperature-triggered fragmentation of the liposome membrane.

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